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For: METHOD OF IDENTIFYING PROPERTIES OF SUBSTANCE WITH RESPECT TO
HUMAN PROSTAGLANDIN D RECEPTORS

CERTIFICATION OF TRANSLATION

Commissioner for Patents
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Sir:

I, Mitsuharu Shimura, residing at, 1-21-10-407, Takadanobaba, Shinjuku-ku, Tokyo 169-0075, Japan, do hereby certify that I am conversant in the English and Japanese languages and that I am a competent translator thereof. I further certify that to the best of my knowledge and belief the attached English translation is a true and correct translation made by me of Japanese Patent Application No. 2000-153172.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of United States Code and that such willful false statements may jeopardize the validity of the application of any patent issuing thereon.

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Respectfully submitted,

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[Title of Invention] Method of Identifying Properties of
Substance with Respect to Human Prostaglandin D Receptors

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[Title of Invention] Method of Identifying Properties of
Substance with Respect to Human Prostaglandin D Receptors

[What is claimed is:]

[Claim 1] A method for identifying a property of a test substance with respect to a human prostaglandin D receptor by correlating an effect of the substance on human CRTH2 with an effect of the substance on the human prostaglandin D receptor.

[Claim 2] The identification method according to claim 1, wherein the effect of the test substance on the human prostaglandin D receptor is selective modulator effect on a human prostaglandin D receptor subtype.

[Claim 3] The identification method according to claim 1 or 2, wherein binding ability, to human CRTH2 or a derivative thereof, of the test substance is used as an index of the property of the substance with respect to the human prostaglandin D receptor.

[Claim 4] The identification method according to claim 1 or 2, wherein in situ agonistic/antagonistic ability, to human CRTH, of the test substance is used as an index of the property of the substance with respect to the human prostaglandin D receptor.

[Detailed Description of the Invention]

[0001]

[Technical Field of the Invention]

The present invention relates to a method of

identifying a substance having specific properties.

[0002]

[Prior Art]

Prostanoids, including prostaglandin, thromboxane, and leukotriene, belong to a family of oxidation metabolites from arachidonic acid, and play an important role in maintaining local homeostasis in the living body. Prostaglandin D₂ (PGD₂), a member of the prostanoid family, is known to be synthesized in various organs including, in mammals, the brain, heart, spleen, lungs, kidneys, bone marrow, stomach, intestine, skin, uterus, and eyeballs, and is known to exhibit various physiological activities (Ujihara, M. *et al.*, Arch. Biochem. Biophys., 260: 521-531, 1988; Ito, S. *et al.*, Prostaglandins, Leukotrienes and Essential Fatty Acids, 37: 219-234, 1989; and references cited therein). In the central nervous system, PGD₂ is considered to participate in, for example, sleep introduction, regulation of body temperature, olfaction, hormone release, inflammation, and analgesia (Negishi, M. *et al.*, Prog. Lipid Res., 32: 417-434, 1993, and the literature cited therein). Furthermore, PGD₂ is known to suppress agglutination of platelets, as well as to cause relaxation of smooth muscle in organs such as the blood vessels, stomach, intestine, and uterus (Giles, H. *et al.*, Prostaglandins, 35: 277-300, 1988, and the literature cited therein). PGD₂ is a typical prostanoid released from a mast cell which plays an important role in immunological reactions. Moreover, PGD₂ is known to participate in formation of

pathological conditions caused by allergy, such as allergic rhinitis and bronchial asthma, through actions such as contraction of bronchial smooth muscle, migration of eosinophiles to an inflammation site, and promotion of release of inflammatory mediators from mast cells, eosinophiles, and basophiles (Negishi, M. *et al.*, *Prog. Lipid Res.*, 32: 417-432, 1993, and the literature cited therein). In addition, when administrated locally, PGD₂ has been demonstrated to have an effect to reduce intraocular pressure (Woodward, D.F. *et al.*, *Eur. J. Pharmacol.*, 230: 327-333, 1993).

[0003]

On the basis of these findings, a substance exhibiting an action on a PGD₂ receptor; for example, a PGD₂-receptor-selective modulator (including an agonist and an antagonist), is considered to be a promising therapeutic drug for various diseases in which PGD₂ participates. For example, physiological actions of PGD₂ suggest that such a substance could be used as a wide range of drugs such as sedatives/sleeping drugs, analgesics, drugs for regulating blood pressure, platelet agglutination inhibitors, drugs for circulatory organs, drugs for suppressing motions of the stomach and intestine, anti-gastric ulcer drugs, therapeutic drugs for allergy, anti-inflammatory drugs, and prophylactic or therapeutic drugs for glaucoma.

[0004]

Presently, PGD₂ has been elucidated to exert its effects

via a specific receptor (Coleman, R. *et al.*, Pharmacol. Rev., 46: 205-229, 1994). However, it is known that the reaction between PGD₂ and its receptor has species specificity, and thus, limitation is imposed on drug screening or evaluation of efficacy of a drug using animal tissue or an animal model (Narumiya, S. *et al.*, Br. J. Pharmacol., 85: 367-375, 1985).

[0005]

Also, a number of pharmacological analyses suggest that, with respect to human and animals, at least two subtypes of PGD₂ receptor mediate various pharmacological actions of PGD₂ (Woodward, D.F. *et al.*, Eur. J. Pharmacol., 230: 327-333, 1993; Fernandes, B. *et al.*, Eur. J. Pharmacol., 283: 73-81, 1995). For example, in human uterus smooth muscle, existence of two subtypes of PGD₂ receptors; one mediating contraction caused by PGD₂ and the other, conversely, mediating relaxation, has been suggested (Fernandes, B. *et al.*, Eur. J. Pharmacol., 283: 73-81, 1995). Therefore, development of a drug which exclusively modulates, among the various actions of PGD₂, a specific action of PGD₂ related to prevention or improvement of pathological conditions requires understanding of the distribution of PGD₂ receptor subtypes in human organs and isolation of the cDNA which codes the receptor subtypes.

[0006]

Recently, after enormous research efforts, a gene which is considered to be a PGD₂ subtype (hereinafter may be referred to as a DP receptor) has been cloned (Boie, Y. *et al.*, J. Biol. Chem., 270: 18910-18916, 1995; Japanese Kokyo

(PCT) Publication No. 10-507930).

[0007]

Currently, studies are being conducted in order to identify, among a variety of physiological activities exhibited by PGD₂, the specific physiological activity or activities in which the DP receptor participates. As a result of such studies, one report describes that analysis on the DP receptor, through expression in the rat brain, has revealed that distribution of the DP receptor does not coincide with the sleep induction sensitive region of PGD₂ (Gerashchenko, D. et al., J. Neurochem., 71: 937-945, 1998). That is, this report suggests the presence of a PGD₂ receptor which is different from the DP receptor, and thus, verification of the presence of such a new receptor and identification of the functions thereof are of keen interest.

[0008]

[Problems to be solved by the Invention]

The subject matter to be handled by the present invention is to find a second human PGD₂ receptor subtype other than the DP receptor, and to provide a method of identifying a substance which acts on the second human PGD₂ receptor subtype and which is useful in the treatment and prevention of a variety of diseases; for example, a substance which acts as a selective modulator (including an agonist and an antagonist).

[0009]

[Means for solving Problems]

Quite unexpectedly, the present inventors have found, during the course of their studies on G-protein-coupling-receptor-like protein human CRTH2 (hereinafter may be referred to simply as human CRTH2) [Nagata, K. et al., J. Immunol., 162: 1278-1286, 1999, and Japanese *Sai-Kohyo* (PCT) Patent Publication WO 97/46677 (this patent publication describes human CRTH2 as "B19")] obtained by genetic cloning by use of human lymphocytes in an attempt to search for its physiological ligands, that human CRTH2 selectively reacts with PGD₂. Further, as a result of comparison between the human CRTH2 and the DP receptor, they have found that the human CRTH2 and the DP receptor react differently with the PGD₂-like substance. Moreover, they have confirmed that a certain compound (e.g., human CRTH2 antibody) can exert selective antagonist activity against human CRTH2, without affecting the DP receptor.

[0010]

Thus, human CRTH2, which initially had been considered to be irrelevant to PGD₂, has surprisingly been concluded to be precisely the aforementioned second human PGD₂ receptor subtype of interest.

[0011]

The present invention is therefore drawn to a method of identifying a substance, which is capable of determining a useful substance having connection with human PGD₂ and which employs a specific property of the human CRTH2; i.e., the capability of acting as a PGD₂ receptor subtype.

[0012]

Thus, in the present patent application, the present inventors provide a method of identifying a test substance (hereinafter may be referred to as the present identification method); specifically, a method of identifying the properties of the test substance with respect to a human prostaglandin D receptor, by determining the correlation between the effect of the test substance on human prostaglandin D receptor (for example, selective modulator effect on the receptor) and the effect of the same test substance on human CRTH2.

[0013]

In the present invention, the term "modulator effect" means any effect that affects the function of the prostaglandin receptor of interest, such as inhibitory effect against the receptor (typically, effect exhibited by an antagonist) or promotion effect (typically, effect exhibited by an agonist), but is not limited thereto.

[0014]

[Embodiments of the Invention]

Embodiments of the present invention will next be described.

A. Human CRTH2

The present identification method is based on the finding that human CRTH2, which is a known human protein, functions as a second human PGD₂ receptor subtype that differs from the DP receptor.

[0015]

Human CRTH2 refers to a human-derived protein registered as "accession number AB008535" in databases DDBJ, EMBL, and GenBank, and the nucleotide sequence of cDNA of human CRTH2 and the amino acid sequence encoded by the sequence are known. The nucleotide sequence and the amino acid sequence are freely available from the aforementioned databases or publications [Nagata, K. et al., J. Immunol., 162: 1278-1286, 1999 and Japanese *Sai-Kohyo* (PCT) Patent Publication WO 97/46677 (this patent publication describes human CRTH2 gene as "B19 gene")].

[0016]

These known data enable preparation of a cloned human CRTH2 protein or a partial peptide thereof, cells expressing human CRTH2, or cell fractions containing human CRTH2, and the thus-prepared products can be used in the present identification method.

[0017]

(1) Preparation of human CRTH2 or a partial peptide thereof

For example, a partial peptide or full-length peptide of human CRTH2 can be obtained through chemical synthesis by means of a known method. More typically, cDNA encoding any of a full-length peptide of human CRTH2, a modified peptide of human CRTH2 in which a portion of amino acids is substituted, deleted, or added, and a partial peptide of human CRTH2 (hereinafter these peptides may be collectively referred to as "human CRTH2-related protein") is prepared;

the resultant cDNA is integrated into an appropriate expression vector; an appropriate host cell is transformed by the expression vector; and a recombinant human CRTH2-related protein can be produced from the resultant transformant.

[0018]

Cloning and recombination of human CRTH2 cDNA can be carried out by means of a standard technique known in the art. The standard technique is described in detail in, for example, Maniatis, T. et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). Gene modification can be carried out by means of a known method; for example, site-specific mutagenesis (Mark, D. F., et al., *Proc. Natl. Acad. Sci. U.S.A.*, 81, 5662 (1984)).

[0019]

In order to express recombinant human CRTH2 in an appropriate host cell, generally, on the basis of known nucleotide sequence data of human CRTH2 cDNA, DNA encoding a human CRTH2-related protein (typically, human CRTH2 cDNA) is cloned in an expression vector. For example, human CRTH2 cDNA can be isolated through known reverse transcription polymerase chain reaction (RT-PCR) by use, as a template, of poly(A) RNA extracted from tissue in which enhancement of expression of a human CRTH2 gene is reported ("PCR Protocols, A Guide to Methods and Applications" edited by Innis, M. A., et al., Academic Press, San Diego, 1990). Examples of human tissue in which expression of a human CRTH2 gene is enhanced include Th2-type T lymphocytes (Nagata, K. et al., J.

Immunol., 162: 1278-1286, 1999), eosinophiles, and basophiles.

[0020]

There may be employed a traditional technique for obtaining full-length human CRTH2 cDNA from, for example, a cDNA library derived from the aforementioned tissue in which human CRTH2 is highly expressed. This can be achieved by use, as a probe, of a fraction of human CRTH2 cDNA amplified through PCR as described above, or chemically synthesized DNA or RNA complementary to the nucleotide sequence of human CRTH2. Such a traditional technique is described in detail in, for example, Maniatis, T. et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

[0021]

An expression vector into which DNA encoding a human CRTH2-related protein, such as human CRTH2 cDNA, is to be integrated preferably has a promoter and an enhancer at the upstream site of a gene to be expressed, and a transcription termination sequence at the downstream site of the gene. Expression of a human CRTH2 gene may be carried out in a direct expression system or in a fusion protein expression system utilizing, for example, a β -galactosidase gene, a glutathione-S-transferase gene, or a thioredoxin gene.

[0022]

Examples of gene expression vectors employing *Escherichia coli* as a host include pQE, pGEX, pT7-7, pMAL, pTrxFus, pET, and pNT26CII. Examples of gene expression

vectors employing *Bacillus subtilis* as a host include pPL608, pNC3, pSM23, and pKH80. Examples of gene expression vectors employing yeast as a host include pGT5, pDB248X, pART1, pREP1, YEp13, YRp7, and YCp50. Examples of gene expression vectors employing a mammal cell or insect cell as a host include p91023, pCDM8, pcDL-SR α 296, pBCMGSNeo, pSV2dhfr, pSVdhfr, pAc373, pAcYM1, pRc/CMV, pREP4, and pcDNAI.

[0023]

These gene expression vectors may be chosen in accordance with purposes of expression of a human CRTH2-related protein. For example, when expression of large amounts of human CRTH2-related proteins is desired, a gene expression vector which can employ *Escherichia coli*, *Bacillus subtilis*, or yeast as a host is preferably selected. When expression of small amounts of human CRTH2-related proteins reliably exhibiting activity is desired, a gene expression vector which can employ a mammal cell or insect cell as a host is preferably selected. As described above, an existing gene expression vector may be selected, but, in accordance with purposes, a new gene expression vector may be produced appropriately, and used.

[0024]

Introduction, into a host cell, of an expression vector containing a gene encoding a human CRTH2-related protein, and transformation of the host cell by the vector can be carried out by means of a routine method. For example, when the host is *Escherichia coli* or *Bacillus subtilis*, a calcium chloride

method or electroporation can be employed. When the host is a mammal cell or an insect cell, calcium phosphate transfection, electroporation, or a liposome method can be employed.

[0025]

When the thus-obtained transformant is cultured by means of a customary method, a desired human CRTH2-related protein is accumulated. A culture medium used in the culture may be appropriately chosen in accordance with the properties of the host cell. For example, when the host is *Escherichia coli*, an LB medium, a TB medium, etc. can be used. When the host is a mammal cell, an RPMI1640 medium, etc. can be used.

[0026]

The recombinant human CRTH2-related protein obtained through the aforementioned procedure is isolated and purified, and can be used as a pure human CRTH2 protein. The human CRTH2-related protein is purified by means of a known technique. Examples of the purification technique include cell solubilization, treatment with a protein precipitant, ultrafiltration, gel filtration, high performance liquid chromatography, centrifugation, electrophoresis, affinity chromatography making use of a specific antibody or a specific ligand, and dialysis. These techniques may be used singly or in combination of two or more. A typical purification method for prostanoid receptor is described in, for example, Ushikubi, F. et al., J. Biol. Chem., 264: 16496-16501, 1989, and purification of the human CRTH2-related

protein may be carried out by means of this method.

[0027]

(2) Cell in which human CRTH2 is expressed, or cell fraction, etc. containing human CRTH2.

Instead of the aforementioned recombinant human CRTH2-related protein, human tissue, a human cell strain, etc. in which natural human CRTH2 is highly expressed may be used in the present identification method. Alternatively, a transformant which has been transformed by a gene encoding the aforementioned human CRTH2-related protein may be used. In such a human tissue, human cell strain, etc., preferably, only human CRTH is expressed, and the DP receptor and other prostanoid receptors are not expressed.

[0028]

A human-CRTH2-expressing cell *per se* can be used in the present identification method. Also, cell fractions containing a human CRTH2 protein (for example, membrane components obtained through supersonic fragmentation treatment and ultracentrifugation) can be obtained from the cell and used.

[0029]

As described in (1) and (2), a cloned human CRTH2-related protein, a cell in which human CRTH2 is expressed, or a cell fraction containing human CRTH2 (hereinafter these protein, cell, and cell fraction may be collectively referred to as "human CRTH2, etc.") can be prepared. The thus-prepared product can be used in the present identification

method.

B. Description of the present identification method

As described above, in the present identification method, the effect of a test substance on human CRTH2, etc. is correlated with the effect of the test substance on a human prostaglandin D receptor. Prior to this correlation, the effect of the test substance on human CRTH2, etc. must be specified.

[0030]

Typical effects of the test substance on the human prostaglandin D receptor include selective modulator effect with respect to a human prostaglandin D receptor subtype.

[0031]

The following descriptions will mainly deal with the selective modulator effect.

As described above, the present identification method is based on the finding that human CRTH2 functions as a second human PGD₂ receptor subtype that differs from the DP receptor, which is the "first human PGD₂ receptor subtype." Therefore, when a test substance is identified as a selective modulator for activation of human CRTH2, the test substance can be identified as a selective modulator with respect to the second human PGD₂ receptor. As described above, the selective modulator with respect to activation of human CRTH2 is identified by evaluating, for example, its selective binding ability with respect to human CRTH2 or its selective effect on the function of human CRTH2, by use of, for example,

a cloned human CRTH2 protein or a partial peptide thereof, a cell expressing human CRTH2, or a cell fraction containing human CRTH2.

[0032]

As described above, the present identification method can employ various molecular forms of human CRTH2. However, in order to evaluate the activity of a selective modulator with respect to human CRTH2, in general, several points must be considered. Firstly, the minimum domain of human CRTH2 required for substantial interaction between human CRTH2 and PGD₂ has not yet been elucidated. Therefore, at present, instead of using a partial peptide of human CRTH2, use of a full-length human CRTH2 protein may be desirable. A human CRTH2 protein derivative—which is formed through substitution or removal of a portion of amino acids of a human CRTH2 protein—may undergo change in ligand selectivity. Therefore, a protein having the same amino acid sequence as that of a naturally occurring human CRTH2 protein may be preferably used. Meanwhile, a sugar chain(s) is bonded to the extracellular site of human CRTH2 which exists as a human membrane protein, and the sugar chain may be necessary for effective interaction between human CRTH2 and PGD₂. Therefore, a host employed for expression of human CRTH2 may be preferably a host cell which enables addition of sugar chain to human CRTH2. In this case, the function of human CRTH2 may vary in accordance with the type of the sugar chain. Therefore, until sufficient data in relation to the

structure of the sugar chain are obtained, a mammal cell may be preferably used as a host cell. When large amounts of human CRTH2 modulators are subjected to screening, the aforementioned modified human CRTH2, partial peptide of human CRTH2, etc. may be advantageously used. Therefore, in accordance with purposes, molecular forms of human CRTH2 employed may be chosen.

[0033]

When a selective modulator with respect to activation of human CRTH2, etc. is identified by means of the present identification method, no absolute limits are imposed on a test substance. Briefly, in the present identification method, a test substance may be a naturally occurring product (including a recombinant protein produced through biotechnological technique) or a chemically synthesized product. When the present identification method is carried out, if necessary, a known labeled or unlabeled ligand (for example, prostanoid such as human PGD₂) may be used.

[0034]

No particular limitation is imposed on the method for identifying the effect of a test substance on human CRTH2, etc., and the effect may be determined by means of any assay known in the art. For example, prostanoid receptor binding assay is described in, Boie, Y. et al., J. Biol. Chem., 270: 18910-18916, 1995.

[0035]

Typical means for correlating the effect of a test

substance on human CRTH2, etc. (for example, a selective modulator effect) with the effect of the test substance on a human prostaglandin D receptor include (1) means in which the binding ability of the test substance with respect to a human CRTH2-related protein is used as an index.

[0036]

When the means (1) is carried out, the binding ability of a test substance with respect to purified human CRTH2 can be determined directly by use of, for example, an apparatus employing surface plasmon resonance as a measurement principle [e.g., Biacore 2000 (product of Amersham Pharmacia)] (see, e.g., a method described in Boris, J. et al., J. Biol. Chem., 272: 11384-11391, 1997). A labeled test substance can be used in the test for directly measuring the binding ability of the test substance with respect to human CRTH2. As described above, the binding ability of a test substance with respect to human CRTH2 can be measured on the basis of, as an index, inhibition or enhancement of the binding of human CRTH2 to a known labeled ligand (e.g., [³H]-labeled PGD₂) (see, e.g., a method described in Boie, Y. et al., J. Biol. Chem., 270: 18910-18916, 1995).

[0037]

If binding ability of a test substance with respect to a human CRTH2-related protein is acknowledged through the means (1), it is very likely that the test substance is a modulator which provides any effect, such as inhibition effect or enhancement effect, on a human prostaglandin D2

receptor.

[0038]

The aforementioned typical means also include (2) means in which the action, as an agonist, of a test substance with respect to a human prostaglandin D receptor is used as an index.

[0039]

The action, as an agonist, of a test substance with respect to a human prostaglandin D receptor can be determined by evaluating in situ activation of human CRTH2 by the test substance, or the effect of the test substance (i.e., inhibition or augmentation) on activation of human CRTH2 by PGD₂. In this case, activation of human CRTH2 can be measured on the basis of, as an index, an increase in the concentration of Ca²⁺ in a host cell (see, e.g., a method described in Boie, Y. et al., J. Biol. Chem., 270: 18910-18916, 1995); enhancement of migration (see, e.g., a method described in Yokomizo, T. et al., Nature, 387: 620-624, 1997); or down modulation of human CRTH2 molecules at a cell surface (see, e.g., a method described in Luttrell, L. M. et al., Science, 283: 655-661, 1999). Examples of the mammal cell strain used as a host when activation of human CRTH2 is measured include K562, Jurkat, HEK293, and CHO.

[0040]

In the case in which the means (2) is carried out, when in situ activity of a human CRTH2 receptor is enhanced by a test substance, the test substance is identified as an

agonist which enhances activity of a human prostaglandin D2 receptor. In contrast, when the receptor activity is reduced by a test substance, the test substance is identified as an antagonist which reduces activity of the human prostaglandin D2 receptor.

[0041]

Selective modulators with respect to human CRTH2 (i.e., a second human PGD₂ receptor) are useful for treating and preventing pathological conditions related to the second human PGD₂ receptor, among various pathological conditions related to PGD₂. Although pathological conditions related to the second human PGD₂ receptor have not yet been fully specified, the aforementioned modulators are expected to find use as drugs for various pathological conditions related to PGD₂, including sedatives/hypnotics, analgesics, blood pressure regulating drugs, platelet aggregation inhibitors, drugs for circulatory organs, drugs for suppressing motions of the stomach and the intestine, anti-gastric ulcer drugs, allergy therapeutic drugs, anti-inflammatory drugs, and drugs for preventing and treating glaucoma.

[0042]

As described above, the properties of a test substance with respect to human prostaglandin D or a human prostaglandin D receptor are identified by correlating the effect of the substance on human CRTH2 (for example, a selective modulator effect) with the effect of the substance on the human prostaglandin D receptor. When the

identification method is used for, for example, screening of drugs, it can greatly contribute to the relevant industry.

[0043]

[Examples]

The present invention will next be described in more detail by way of Examples, which should not be construed as limiting the invention thereto.

[0044]

[Example 1] Preparation of a cell in which human CRTH2 is expressed

Human CRTH2 gene expression plasmid pRc/B19 was prepared by means of a method described in detail in Nagata, K. et al., J. Immunol., 162: 1278-1286, 1999 and Japanese *Sai-Kohyo* (PCT) Patent Publication WO 97/46677. A transformant into which full-length human CRTH2 cDNA is integrated is deposited as B19 cDNA in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (deposit number: FERM P-15616), and B19 cDNA can be used after approval is obtained from the depositor. When B19 cDNA is digested with restriction enzymes HindIII and XbaI, and is subcloned into the HindIII/XbaI site of plasmid pRc/CMV (product of Invitrogen), the same expression plasmid as the aforementioned pRc/B19 can be prepared. Through electroporation, the pRc/B19 and control plasmid pRc/CMV were genetically introduced into K562 cells, a human erythroblastic leukemia cell strain, and were subjected to selective culture for two to three weeks in the presence of

400 µg/ml of geneticin (product of SIGMA). Among the K562 cells into which the pRc/B19 had been genetically introduced, the survived cells were added to a 96-well microplate (0.3 cells per well), and then cultured for two to three weeks. The proliferated cell clones were subjected to fluorescence staining by use of an anti human CRTH2 antibody (Nagata, K. et al., J. Immunol., 162: 1278-1286, 1999), and clone KB8, in which human CRTH2 was highly expressed, was obtained. The K562 cells into which the control pRc/CMV had been genetically introduced were selected by use of geneticin, and then culture of the resultant cells was continued while polyclones were present (hereinafter the cells will be referred to as "K562/neo").

[0045]

[Example 2] Preparation of comparative cell in which DP receptor is expressed

Human DP receptor cDNA was cloned through RT-PCR on the basis of the nucleotide sequence of human DP receptor cDNA described in Boie, Y. et al., J. Biol. Chem., 270: 18910-18916, 1995 and Japanese *Kohyo* (PCT) Patent Publication No. 10-507930. Specifically, the cDNA was prepared by use, as a template, of poly(A)+RNA (product of Clontech) of the human small intestine in which the human DP receptor is highly expressed, and by use of oligo (dT) primer (product of Pharmacia) and MMLV reverse transcriptase (product of Pharmacia). Subsequently, the coding region of the human DP receptor was amplified through PCR (35 heat cycles, each

cycle consisting of heating at 94°C for 1 minute, at 68°C for 1 minute, and at 72°C for 1 minute) by use of a forward primer (5'-CTTCCGAAGCTTTCACTCCAGCCCTCTGCTCCCG: SEQ No. 1) containing a restriction enzyme HindIII site, and a reverse primer (5'-GTTCTTTTCTATAAAATGTGACATATTCCTCAGCTTACC: SEQ No. 2) containing an XbaI site. The resultant PCR product was digested with HindIII and XbaI, and subcloned into the HindIII/XbaI site of pRc/CMV. The thus-prepared human DP receptor expression plasmid was introduced into a K562 cell in a manner similar to that described in Example 1, and subjected to selection by use of geneticin and to cloning. The proliferated clones were subjected to the below-described [³H]PGD₂ binding assay, to thereby obtain clone KD36 in which the DP receptor is highly expressed.

[0046]

[Example 3] [³H]PGD₂ binding assay

Each of KB8 cells, KD36 cells, and K562/neo cells was resuspended in Hank's balanced salt solution (HBSS, product of Gibco BRL) so as to attain a concentration of 3×10^7 cells/ml. The resultant suspension (0.1 ml) was placed in a 0.5-ml microtube, and then cooled on ice. Subsequently, 1 nM of [³H]PGD₂ (product of Amersham) which had been diluted with HBSS was added to the suspension, to thereby allow reaction to proceed on ice for one hour. The reacted cells were placed carefully onto RPMI1640 medium (1 ml) containing 1 M sucrose and 10% fetal bovine serum, the medium having been placed in an 1.5-ml microtube and cooled by ice, and

subjected to centrifugation (10,000 revolutions, three minutes) by use of a micro-centrifuge. After the supernatant was aspirated from the tube such that the mixture (about 0.1 ml) remained in the tube, the mixture was further subjected to centrifugation (10,000 revolutions, one minute) such that the reaction mixture did not remain on the tube wall, and subsequently the supernatant was removed as carefully as possible so as to avoid removing the cells. The radiation activity of the cells bound to [^3H]PGD₂ was measured by use of a liquid scintillation counter. The radiation activity of the cells when measured, in a manner similar to that described above, in the presence of unlabeled PGD₂ (concentration: 200 times or more that of [^3H]PGD₂) was used as an index of non-specific binding. As a result, as shown in Fig. 1, the specific binding of [^3H]PGD₂ to K562/neo is not observed. In contrast, the specific binding of [^3H]PGD₂ to KB8 or KD36 is observed. In this measurement system, anti CRTH2 antibody BM7 (Nagata, K. et al., J. Immunol., 162: 1278-1286, 1999 and Nagata, K., et al.) selectively inhibited the binding of [^3H]PGD₂ to KB8 in a concentration-dependent manner. The results show that this method can identify a selective modulator with respect to human CRTH2, which does not act on the DP receptor.

[0047]

[Example 4] Intracellular calcium assay

Each of KB8 cells, KD36 cells, and K562/neo cells was suspended in RPMI1640 medium containing 10% fetal bovine

serum so as to attain a concentration of 5×10^6 cells/ml, and cultured at 37°C for one hour in the presence of 5 μ M of Fura-2AM (product of Dojindo). After completion of culturing, the cells were washed three times with HBSS through centrifugation, to thereby remove free Fura-2AM. Subsequently, the resultant cells were resuspended in HBSS containing 0.1% bovine serum albumin so as to attain a concentration of 10^6 cells/ml. The resultant suspension was placed in a quartz cell (volume: 0.4 ml), and the cell was set in spectrophotometer LS50B (product of PerkinElmer). After a sample was added to the suspension, the time-course change of the ratio of fluorescence intensities (510 nm) at excitation wavelengths of 340 nm and 380 nm was obtained through calculation by use of FL-Winlab software (product of PerkinElmer). In the case of K562/neo, the concentration of calcium in the cells did not vary significantly by treatment with PGD₂ (0.25-250 nM). In contrast, in the case of KB8 or KD36, the concentration of calcium in the cells increased considerably by treatment with PGD₂ (some nM). In this measurement system, anti-CRTH2 antibody BM7 exhibited selective antagonistic activity with respect to human CRTH2 (Fig. 2). These results show that this method can identify a selective modulator with respect to human CRTH2, which does not act on the DP receptor.

[0048]

[Example 5] Binding activity of various prostanoids with respect to human CRTH2

[³H]PGD₂ binding assay described in Example 3 was carried out in the presence of various unlabeled prostanoids (products of Cayman), and the binding affinity of human CRTH2 to various prostanoids was evaluated. As a result, the order of the degree of the binding affinity of prostanoids to human CRTH2 was determined as follows: PGD₂ ≥ 13,14-dehydro-15-keto PGD₂ >> prostaglandin J₂ (PGJ₂) > 15-deoxydelta 12,14-PGJ₂ ≥ prostaglandin E₂ (PGE₂) = prostaglandin A₂ (PGA₂) >> thromboxane B₂ (TXB₂) (Fig. 3). For comparison, the order of the degree of the binding affinity of prostanoids to the DP receptor was determined as follows: PGD₂ = PGJ₂ >> PGE₂ > 13,14-dehydro-15-keto PGD₂ > 15-deoxydelta 12,14-PGJ₂ ≥ TXB₂ ≥ PGA₂. These results show that the degree of the binding affinity of human CRTH2 to PGD₂ is equal to that of the binding affinity of the DP receptor to PGD₂, and that a great difference exists between human CRTH2 and the DP receptor in the degree of binding affinity to other prostanoids, such as 13,14-dehydro-15-keto PGD₂.

[0049]

[Example 6] Down modulation of human CRTH molecules by selective agonist

KB8 cells were suspended in RPMI1640 medium containing 10% fetal bovine serum so as to attain a concentration of 10⁶ cells/ml, and cultured at 37°C for one hour in the presence of a test sample. After completion of culturing, the cells were washed twice with HBSS through centrifugation, to thereby remove cell-free part of the sample. Subsequently,

the resultant cells were resuspended in HBSS containing 0.5% bovine serum albumin and 0.05% NaN₃, and the resultant suspension was subjected to fluorescence staining by use of anti CRTH2 monoclonal antibody BM16 and phycoerythrin-labeled anti rat immunoglobulin antibody. The average fluorescence intensity of the stained cells was measured by use of a flow cytometer (product of Becton Dickinson), and the fluorescence intensity of the cells treated with the sample was compared with that of non-treated cells (control). The results are shown in Fig. 4.

[0050]

As shown in Fig. 4, PGD₂ and 13,14-dehydro-15-keto PGD₂, which are selective agonists, induce down modulation of CRTH2 significantly as compared with the control. In contrast, PGE₂ exhibiting no agonistic activity, does not exert such an effect. The results show that this method can be used to identify a selective agonist with respect to CRTH2.

[0051]

[Effects of the Invention]

The present invention provides a method for identifying a substance (for example, a selective modulator such as an agonist or antagonist) which acts on a newly found second human PGD₂ receptor subtype that differs from the DP receptor, and is useful for treating or preventing various diseases.

[0051]

[Sequence Listing]

SEQUENCE LISTING

<110> BML, INC.

<120> Method of identifying property of substance against
human prostaglandin D receptor

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39

[Brief Description of the Drawings]

[Fig. 1] Fig.1 shows the results of an assay,
performed in accordance with the method described in Example
3, investigating the [³H]PGD₂ binding ability of cells
expressing human CRTH2 (KB8) or DP receptor (KD36).

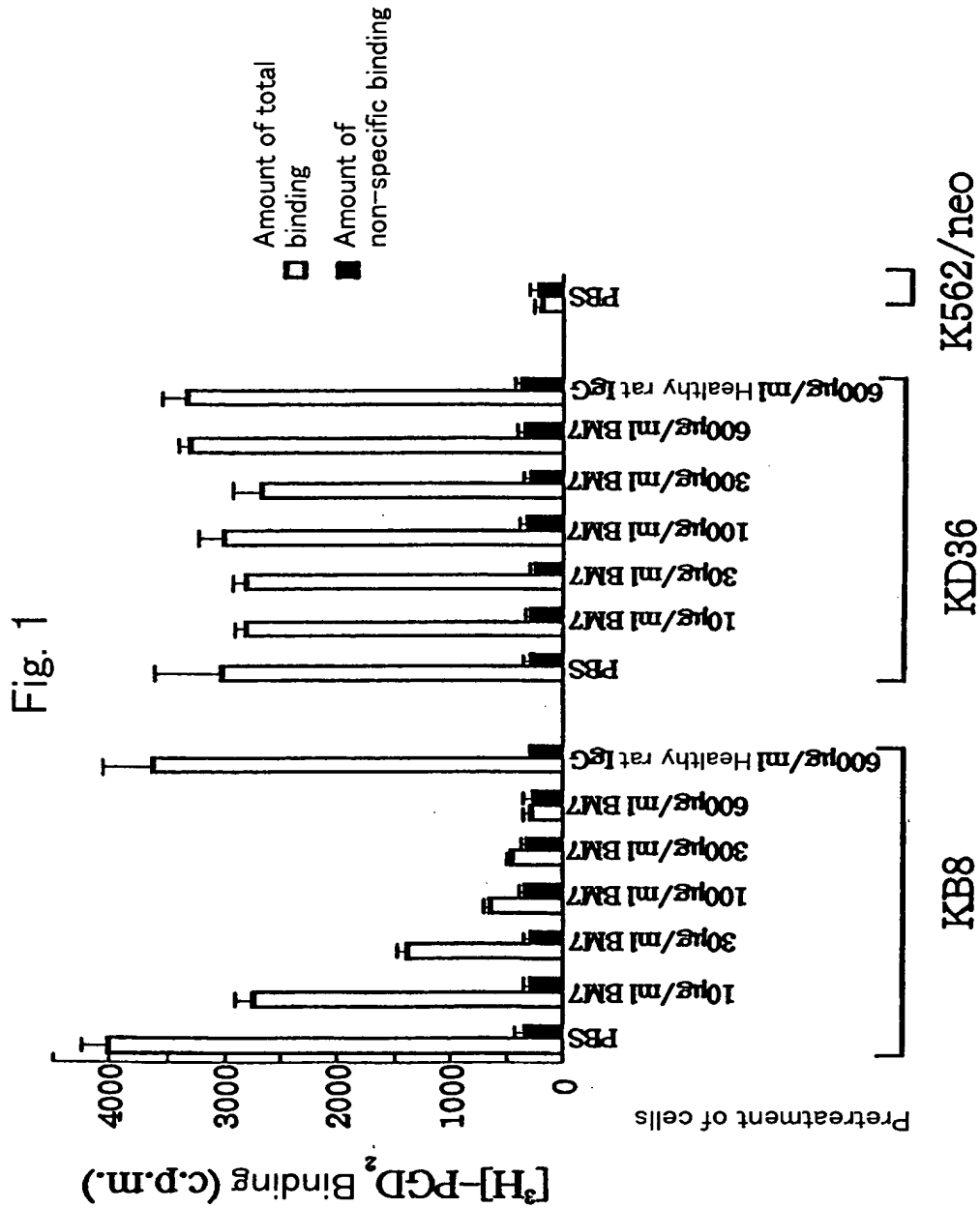
[Fig. 2] Fig. 2 shows the results of an intracellular calcium assay, performed in accordance with the method described in Example 4, investigating the reaction, with PGD₂, of cells expressing human CRTH2 (KB8) or DP receptor (KD36).

[Fig. 3] Fig. 3 shows the results of an assay, performed in accordance with the method described in Example 5, investigating the ligand selectivity of human CRTH2 and that of DP receptor.

[Fig. 4] Fig. 4 shows the results of a test, performed in accordance with the method described in Example 6, regarding down modulation from a cell surface, induced by the selective agonist of human CRTH2.

[Document] Drawings

[Fig.1]



[Fig.2]



Fig. 2

Pretreatment
of cells

PBS

20 min at
room temperature

BM7

500 μ g/ml

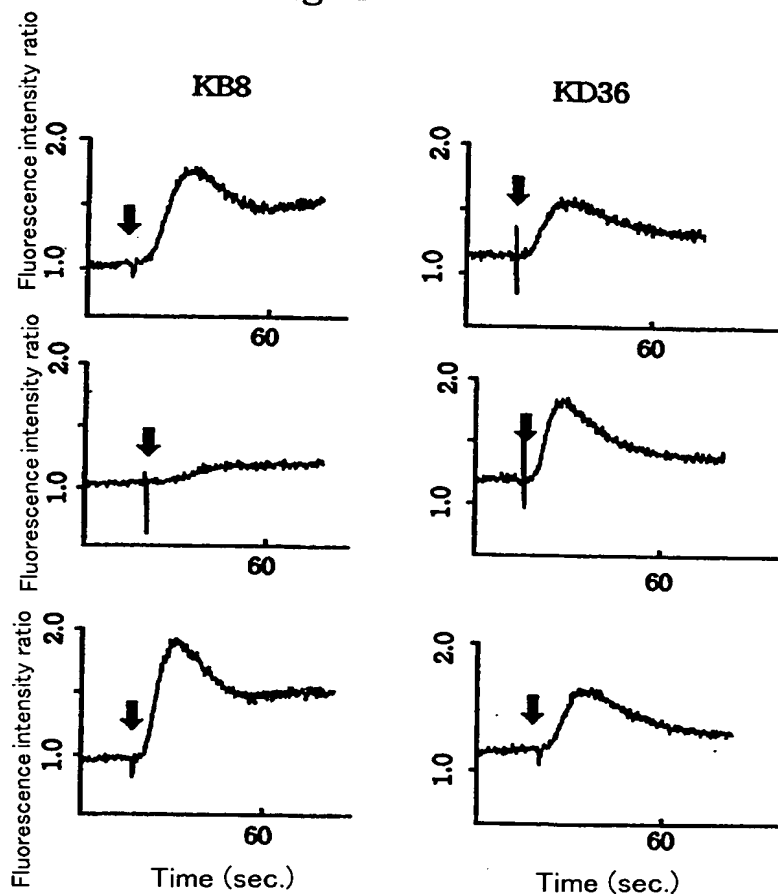
20 min at
room temperature

Healthy rat

IgG

500 μ g/ml

20 min at
room temperature

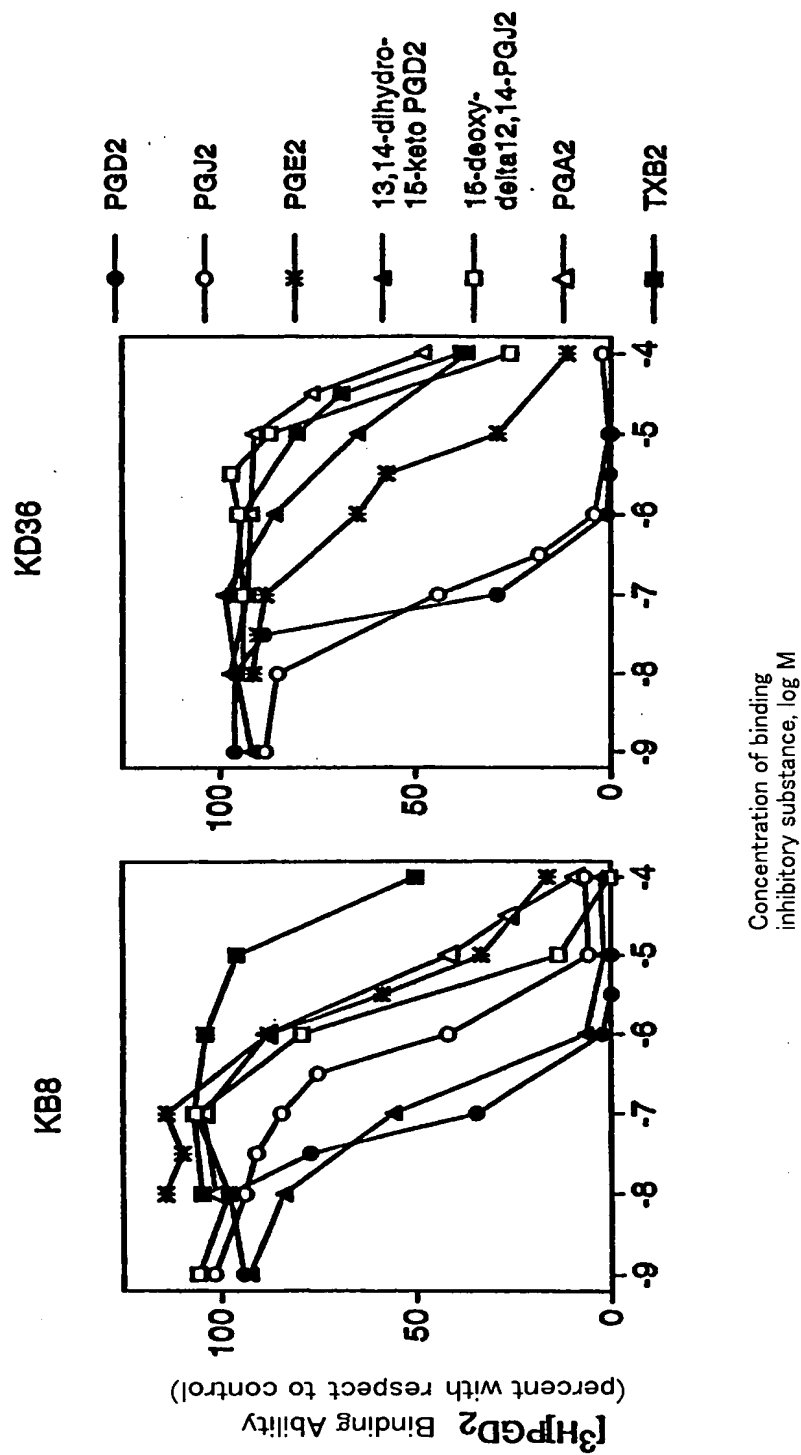


* At the point in time indicated by
the black arrow in each Fig.,
PGD₂ (final concentration: 25 nM)
was added to the reaction mixture.

[Fig. 3]



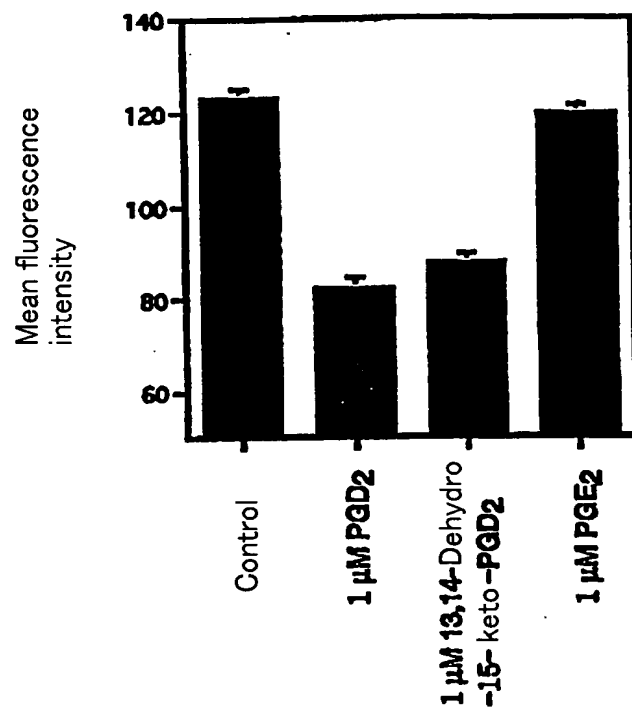
Fig. 3



[Fig. 4]



Fig. 4



[Document] Abstract

[Abstract]

[Problems] To find a second human PGD₂ receptor subtype other than the DP receptor, and to provide a method of identifying a substance which acts on the second human PGD₂ receptor subtype and which is useful in the treatment and prevention of a variety of diseases.

[Solving Means] The inventors have found that the above-described problems can be solved by providing a method for identifying a property of a test substance with respect to a human prostaglandin D receptor by correlating an effect of the substance on human CRTH2 with an effect of the substance on the human prostaglandin D receptor.

[Elected Figure] None